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The Transmission of the Rous Filterable Agent to Chemically Induced Tumours

BY

EDWARD MELLANBY

Field Laboratory, University of Sheffield

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THE TRANSMISSION OF THE ROUS FILTERABLE AGENT TO CHEMICALLY INDUCED TUMOURS.

Edward Mellanby.

Field Laboratory, University of Sheffield.

(Plate XLVII.)

In the Report of the British Empire Cancer Campaign for 1934 I referred to experiments in which it had proved impossible to show the presence of a filter-passing agent in cancerous growths of fowls induced by dibenzanthracene and tar. This result was not in agreement with that previously published by McIntosh (1933), who demonstrated the presence in tar-induced tumours of a filterable agent comparable to the well known agent of the Rous and other bird tumours. I pointed out that if chemically induced tumours in fowls contain a filterable active agent, it appeared to be exceptional and not the rule. In 1928 Sturm and Murphy had also failed to obtain any evidence of the presence of a filterable active agent in tar tumours of fowls. The suggestion has been made that the positive results obtained by McIntosh may have been due to the fact that some of the birds he used carried in their tissues an infective agent of the bird leukæmia type at the time they were bearing the tar tumours, and that it was this adventitious agent which really stimulated the formation of new tumours and not the specific product of the tar tumours. Even on the basis of this explanation the subject seemed of interest. I proceeded, therefore, to see whether the carcinogenic agent produced in a Rous sarcoma could pass to a chemically induced tumour in the same bird, and if so, whether the cancerous growths resulting from such a filterable agent would simulate the chemically induced or the Rous sarcoma as regards structure and metabolic activity. In the Report of the British Empire Cancer Campaign for 1935 I gave a resumé of the results obtained. It was then stated that in the case of birds bearing at the same time both dibenzanthracene and Rous tumours, cell-free filtrates made from the dibenzanthracene tumours and injected into fowls resulted in new tumours and that these, both structurally and according to metabolic tests, had the properties of a Rous sarcoma and not of the original dibenzanthracene tumour.

These experiments demonstrated that the filterable agent of a Rous sarcoma could pass readily into a chemically induced sarcoma which itself had no filterable agent so far as could be detected by the methods used.

The present publication supplies the evidence upon which these statements were made. It suggests further that the presence of the Rous agent in the dibenzanthracene tumour does not alter the character of the latter, so that although cell-free filtrates induce Rous tumours, an injection of the cells of the infected dibenzanthracene tumour produces another dibenzanthracene tumour and not one of the Rous type.

More recently some experiments have been made in which fowls carried three kinds of tumours, dibenzanthracene tumours in one breast, tar tumours in the other breast and Rous tumours in each leg. One of these experiments is reported and evidence is given of the passage of the Rous agent to the tar tumour.

Method of experiment.

The method adopted was to inject an inoculum of cells of a chemically induced tumour, in most of the experiments of the dibenzanthracene type, into the breast muscles of fowls. At varying stages of development of these tumours cells of a Rous sarcoma were injected into both legs of each fowl. In the course of time the fowls had a dibenzanthracene tumour in one or both breasts and a Rous sarcoma in each leg. When the fowls were killed or died, cell-free filtrates of the dibenzanthracene tumours in the breasts were made and injected into normal fowls. In other fowls the *cells* of the dibenzanthracene tumours were injected. From any subsequent tumours which developed, injections of cells and cell-free filtrates were made for a varying number of generations in different experiments.

Throughout the work here reported, the preparation of the cell inocula and cell-free filtrates and the amounts injected have been kept approximately constant with two exceptions. For the filtrates, about 1 part of tumour tissue was ground up with silver sand and extracted with 9 parts of normal saline made with tap water. This extract was centrifuged and then filtered through paper pulp, 1 c.c. doses of the filtrate being inoculated into each breast of the fowls. When cells were used 0·05 c.c. of scissors-minced tissue was used for inoculation into each breast. In one of the early experiments (1934) recorded below (exp. A), 2 c.c. of the cell-free filtrate were injected. It might have been better to have injected this amount in the other experiments instead of 1 c.c., since the positive results in this case were two out of three, whereas usually the proportion of successes was less.

The methods whereby differentiation of the two types of tumours—dibenzanthracene and Rous—was made will now be mentioned.

(1) In my experience, a cell-free filtrate of an ordinary dibenzanthracene or tar tumour of a fowl does not produce another tumour on inoculation as does that of a Rous tumour.

(2) The appearances of a dibenzanthracene or tar sarcoma and a Rous sarcoma, macroscopically and microscopically, are usually different; the first two are more cellular and solid, the Rous tumour is mucilaginous and of loose texture. The diagnosis of these types of tumours by their histological appearance is illustrated in figs. 1-4 (pl. XLVII). A fowl carried a dibenz-

anthracene tumour in the breast and a Rous tumour in each leg. The structure of the former is seen in fig. 1, of the latter in fig. 2. *Cells* of the dibenzanthracene tumour were inoculated into another fowl and gave rise to a tumour of the same type (fig. 3). On the other hand, a *cell-free filtrate* of the same dibenzanthracene tumour (fig. 1) gave rise to a tumour of typically Rous appearance (fig. 4).

Occasionally, however, a Rous tumour of the slow-growing type is found which is more cellular and more difficult to differentiate from the chemically induced tumours.

(3) It was hoped that a comparison of the metabolic properties of the tumours might assist in distinguishing them, but it is now doubtful whether this method is any better than the histological examination, although of course it provides additional support for diagnostic purposes.

It is true that the metabolic characters of these two tumours are sufficiently different to allow classification when typical tumour tissue of each type is examined. Examined by the Warburg method, with the Dickens-Šimer apparatus (1930, 1931), the greatest and most constant metabolic difference is the glycolytic power of each estimated in terms of the dried weight. For the Rous sarcoma of typical structure the glycolytic action $Q_G^{O_2}$ per mg. dried tissue per hour varies between 7 and 9. The corresponding figure for the fowl dibenzanthracene tumour tissue is 5-6. The other two figures given by the Warburg technique—$Q_{CO_2}^{O_2}$, the carbon dioxide produced by oxidation per mg. dried weight per hour, and Q_{O_2}, the oxygen uptake per mg. dried weight per hour—vary widely in different tumours of the same type, and the range is too similar to provide a measure of differentiation, although the more typical and rapidly growing the Rous tumour, the greater is the oxygen uptake as compared with that of the dibenzanthracene tumour.

It is of interest to note that the percentage of dried substance in Rous tumour is usually much smaller than that of the fowl dibenzanthracene tumour—an average of 10-11 per cent. as compared with the 18 per cent. in the case of the dibenzanthracene tumour tissue. This figure, obtained merely by drying tissue slices of the respective tumours, is in itself a good indication of the type of tumour, but may fail in differentiating the uncommon, hard, slow-growing Rous tumour from the typical dibenzanthracene tumour. These various methods, the mode of propagation of the tumours, the gross and histological appearance, the relative metabolic activity and the percentage dried weight, have been used to differentiate the types of tumour produced from the experimental cell and cell-free-filtrate inoculations.

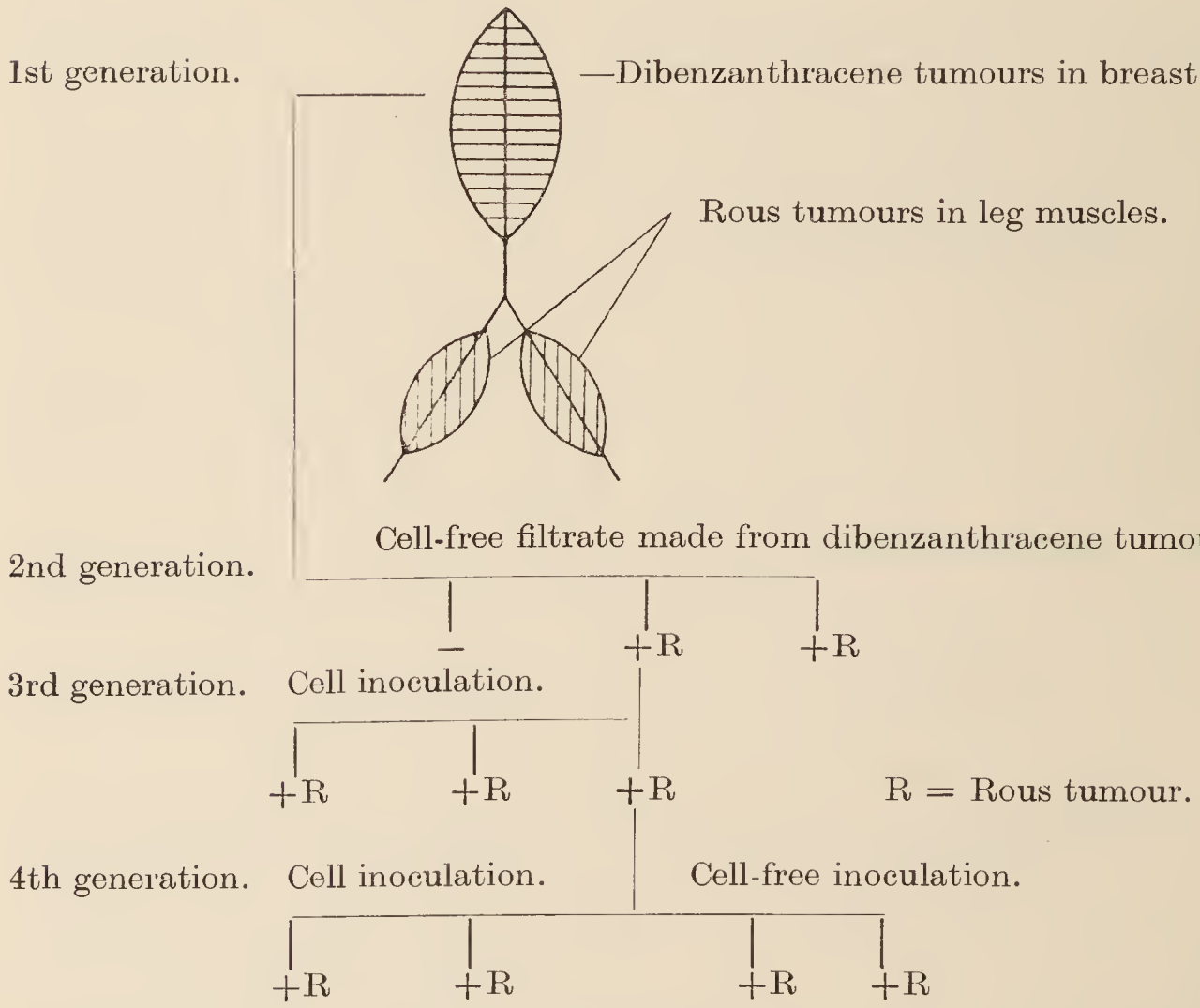
Experimental results.

The following may be taken as an example of one of the early experiments :—

Experiment A. A fowl was inoculated into both breast muscles with dibenzanthracene tumour tissue (FD/2) : 11 days later there were two palpable tumours ; after another 13 days, when the tumours were large, both legs were inoculated with Rous sarcoma cells. All four tumours were growing when the fowl died, 38 days after the beginning of the experiment. A paper-pulp filtrate (cell-free) was made of the breast (dibenzanthracene) tumours : this, on inoculation into three fowls, produced tumours in two of them. These tumours

were further propagated both by cell and cell-free inoculation, resulting in each case in typical Rous tumours.

It is of interest to note that the dibenzanthracene tumour (FD/2) grew much more slowly at the time of this experiment in 1934 than in later years. This explains why there was a longer interval after inoculation between the dibenzanthracene and the Rous products in this than in later experiments.



Experiment A.

Here, then, is an instance of a dibenzanthracene tumour containing Rous filterable agent, transmitted to it from Rous sarcomata in the leg, which was propagated, like Rous tumours, on the injection of a cell-free filtrate. In this instance the cells of the original dibenzanthracene tumour were not inoculated. In that case, from analogy with many other experiments (see expts. B and C), a dibenzanthracene tumour and not a Rous tumour would have grown. Figs. 1-4 illustrate such an experiment.

Experiment B. The following experiment also shows that the original dibenzanthracene tumour retains its own properties on cell propagation, even when it contains sufficient Rous filterable agent to give rise to a tumour of the Rous type :

A fowl was inoculated, into the right breast only, with dibenzanthracene tumour cells (FD/2). Seven days later, Rous tissue was injected into both legs. After a further period of 11 days the fowl, now bearing fairly big tumours in the right breast and both legs,

HISTOLOGY OF TUMOURS IN A TYPICAL EXPERIMENT

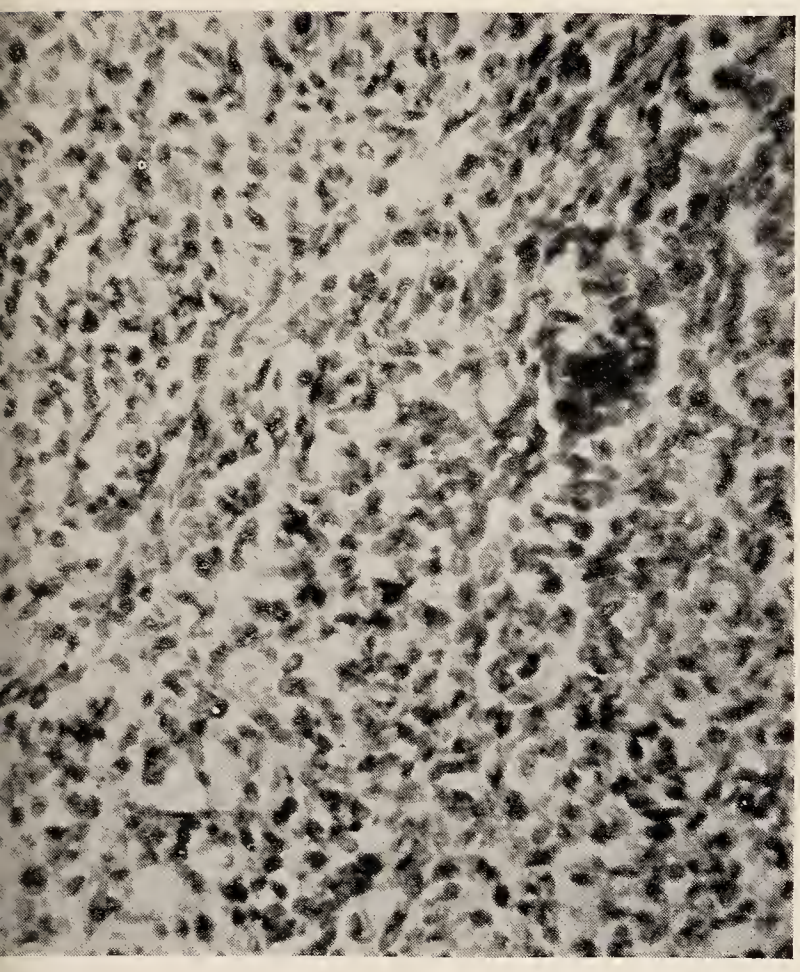


FIG. 1.—Typical dibenzanthracene tumour of a fowl. ×350.

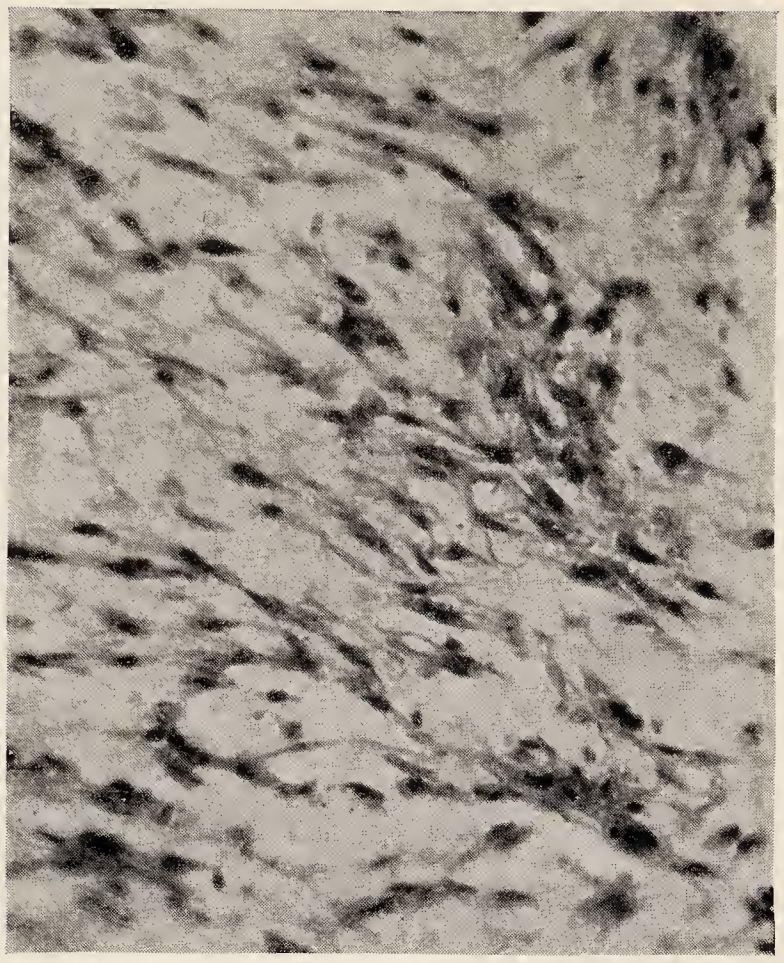


FIG. 2.—Typical Rous tumour of a fowl. ×350.

These tumours were present in the breast and leg respectively of the same fowl.

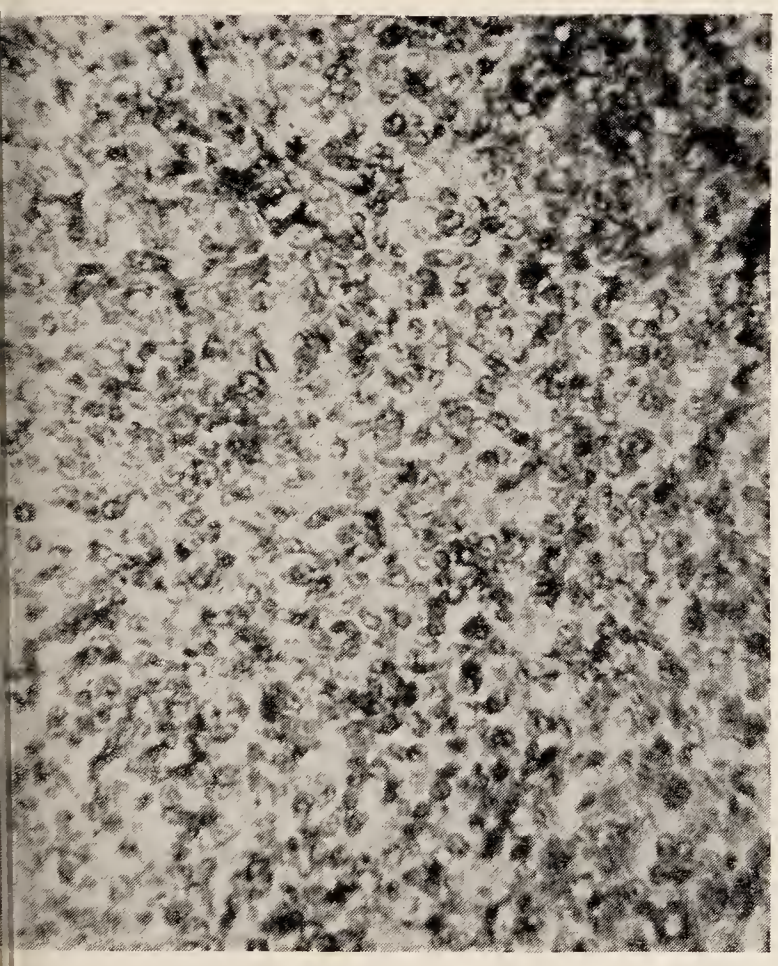


FIG. 3.—Dibenzanthracene tumour in a fowl produced by a cell inoculum from the tumour represented in fig. 1. ×350.

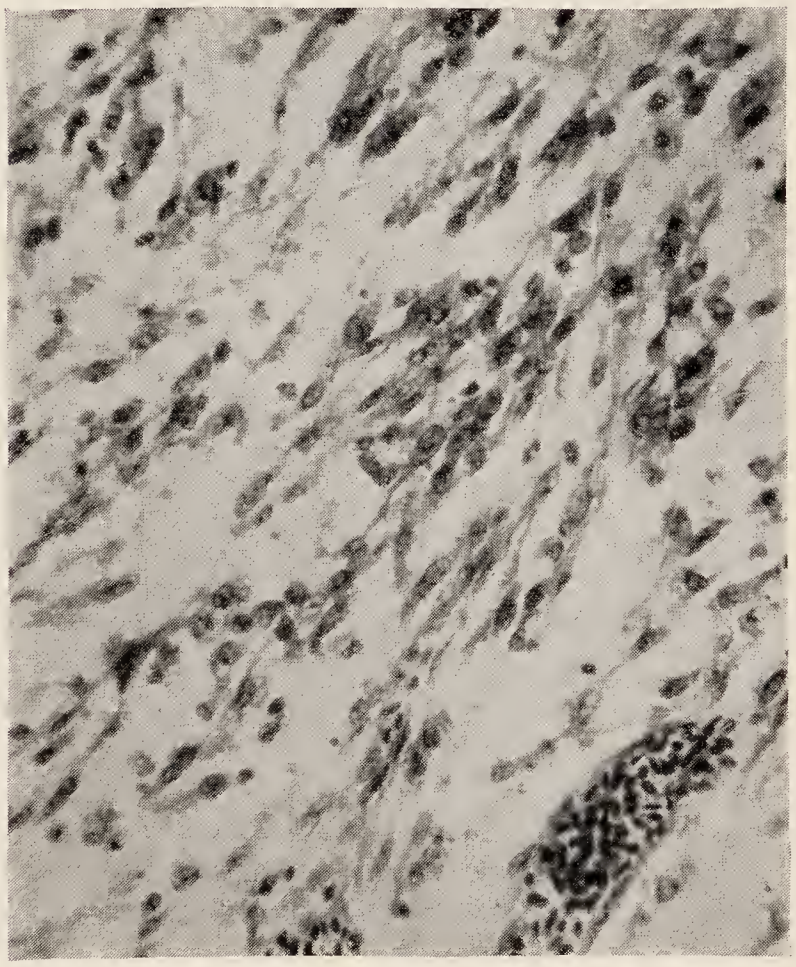
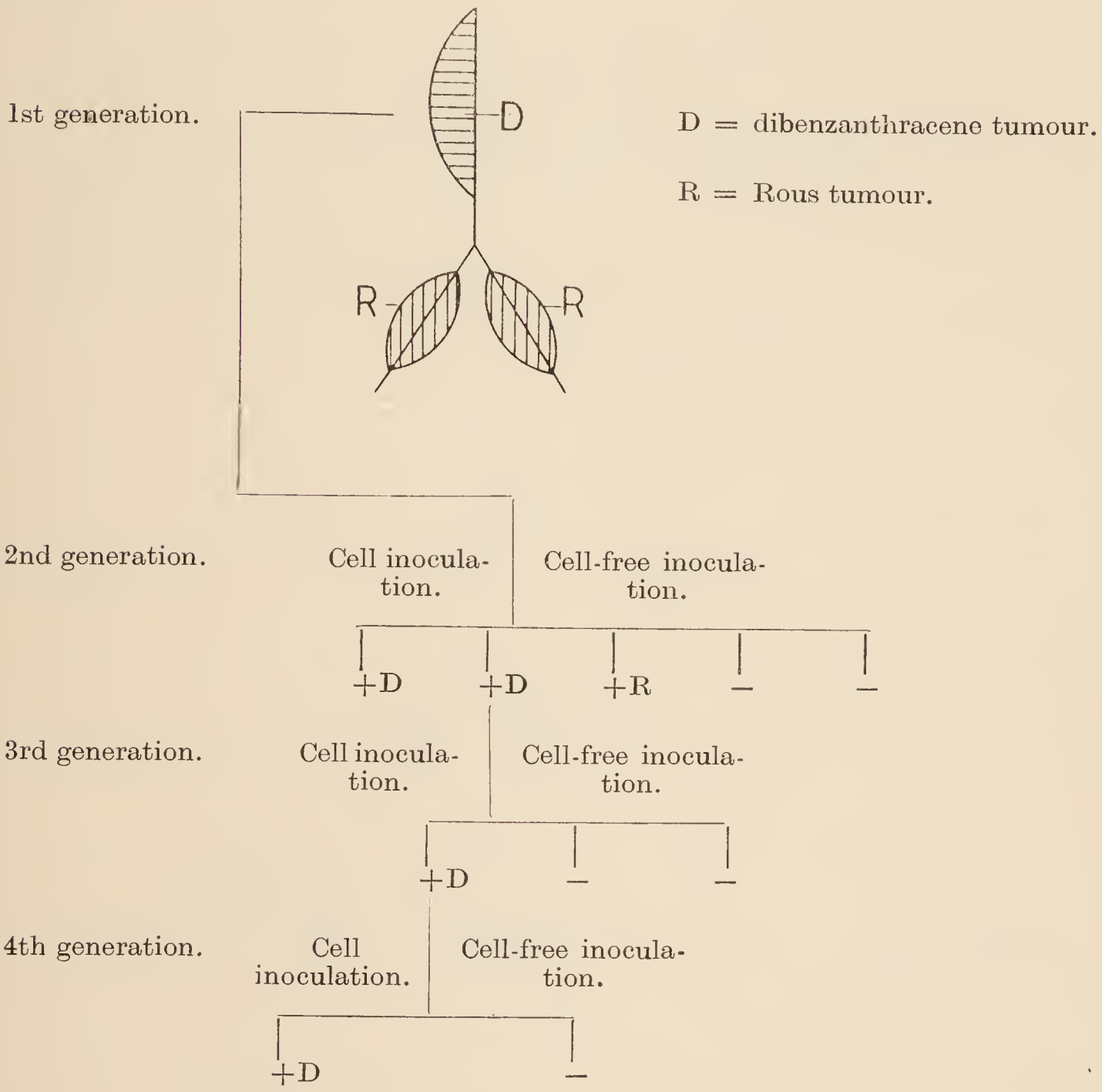


FIG. 4.—Rous tumour in a fowl produced by a cell-free inoculum made from the dibenzanthracene tumour represented in fig. 1. ×350.

was killed. Cells and cell-free filtrate from the breast tumour were inoculated into other fowls as below.



EXPERIMENT B.

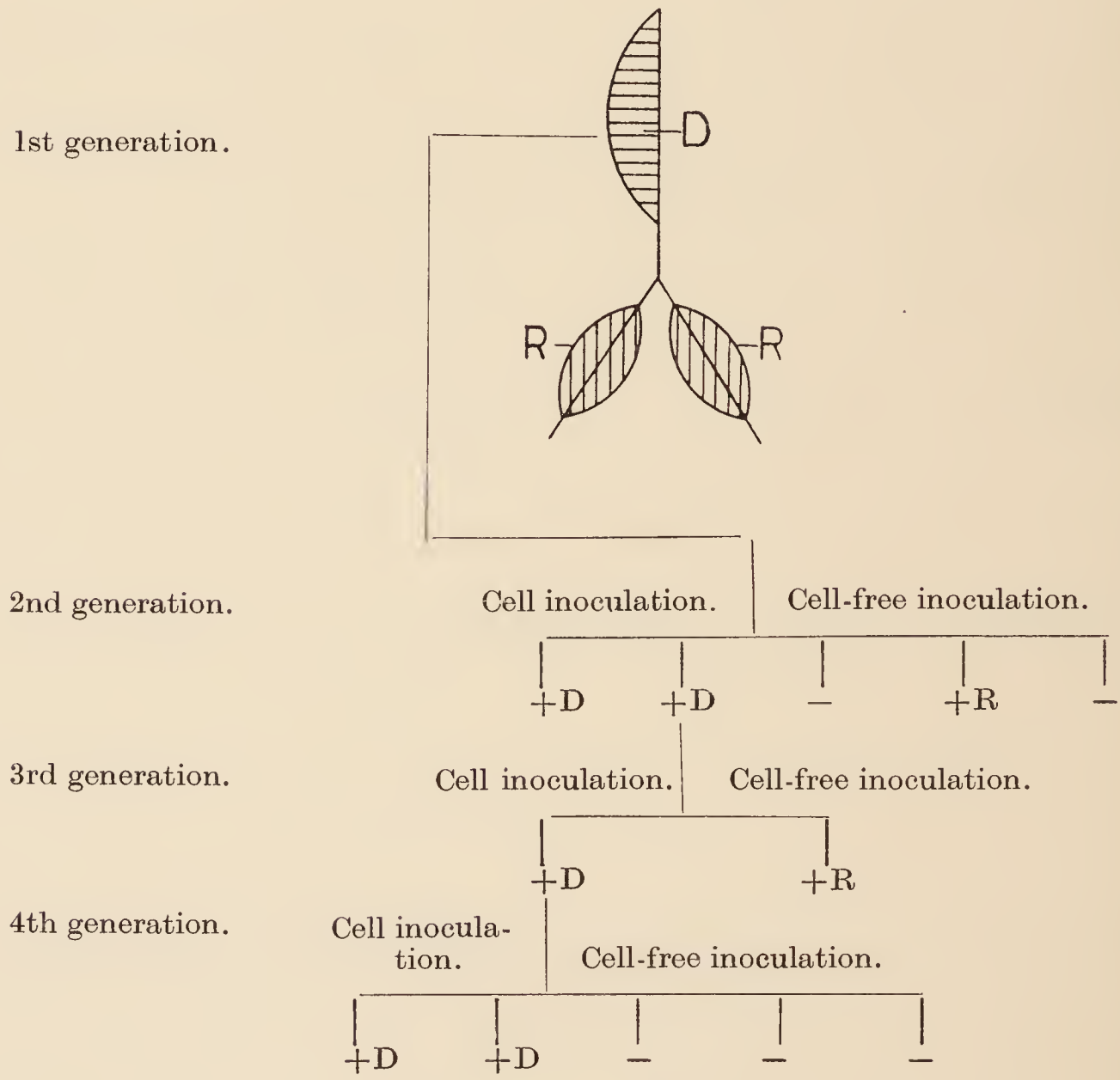
In this case a typical Rous tumour was produced, in 1 bird out of 3, by inoculation with a cell-free filtrate of the original dibenzanthracene tumour ; the cells themselves, however, produced dibenzanthracene tumours in 2 birds injected.

It is usually only possible to get evidence of the presence of the Rous filterable agent in the dibenzanthracene tumour when a Rous tumour is growing in the same bird. In two experiments, however, there was evidence of the presence of the Rous agent in the second generation of both dibenzanthracene and tar tumours, *i.e.* tumours which had grown in birds which themselves had no accompanying Rous tumour. This is illustrated in the next experiments.

Production of Rous tumours from the second generation of chemically induced tumours by cell-free filtrates.

In the first experiment the tumour from which the cell-free filtrate produced a Rous sarcoma was of the dibenzanthracene type.

Experiment C. A fowl was inoculated into the right breast muscle with tissue from a dibenzanthracene tumour (FD/2). Eight days later Rous tissue was inoculated into both legs. The fowl was killed 13 days after the second inoculation, when it had tumours in the right breast and in both legs. Cells and cell-free filtrates of the dibenzanthracene tumour were then inoculated into other fowls, and the experiment proceeded as follows.



Experiment C.

It will be seen in this experiment that, in addition to the Rous tumour produced by a cell-free filtrate of the original dibenzanthracene tumour, another Rous tumour was produced by a cell-free inoculation of a second generation dibenzanthracene tumour, although this tumour had never itself been in association with a Rous sarcoma.

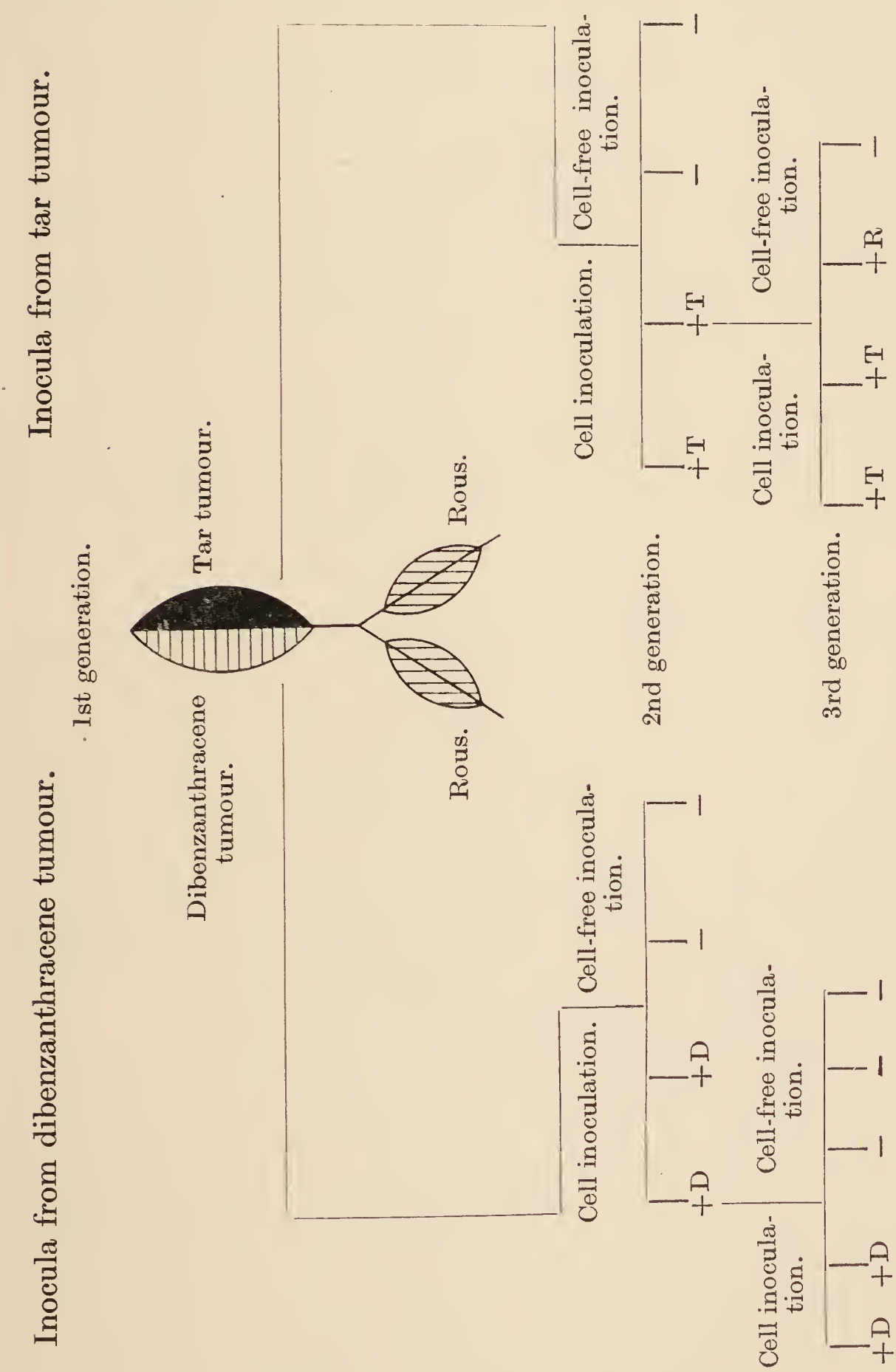
In the following experiment an active cell-free filtrate was obtained from the second generation of a *tar* tumour. No active

filtrate was obtained from either the first or second generation of the *dibenzanthracene* tumour in the right breast of the same fowl.

Experiment D. A fowl was inoculated on the same date as follows.

Right breast—0·05 c.c. dibenzanthracene tumour tissue.
Left ,, —0·05 c.c. tar tumour tissue.
Both legs (intramuscularly)—0·05 c.c. Rous tumour tissue.

Eighteen days later, when the fowl was killed, there were large



EXPERIMENT D.

tumours in both breasts and fairly big tumours in both legs. There were no metastases. Cell suspensions and cell-free filtrates

were made from both the dibenzanthracene and tar tumours and injected into other fowls.

It will be seen (p. 453) that, whereas the cell-free filtrate of the first generation of the tar tumour gave negative results on inoculation, that of the second generation produced a Rous sarcoma.

In this experiment the following points may be noted.

1. The original bird carried three types of tumours, Rous in the legs, a dibenzanthracene tumour in the right breast and a tar tumour in the left breast.

2. Inocula of cells and cell-free filtrates from both the tar tumour and the dibenzanthracene tumour were injected into other fowls.

3. Injection of a cell-free filtrate of the original tar tumour was negative whereas an injection of a cell-free filtrate made from the second generation of tar tumour produced a Rous sarcoma.

4. A cell-free filtrate of the dibenzanthracene tumour from both first and second generations was negative.

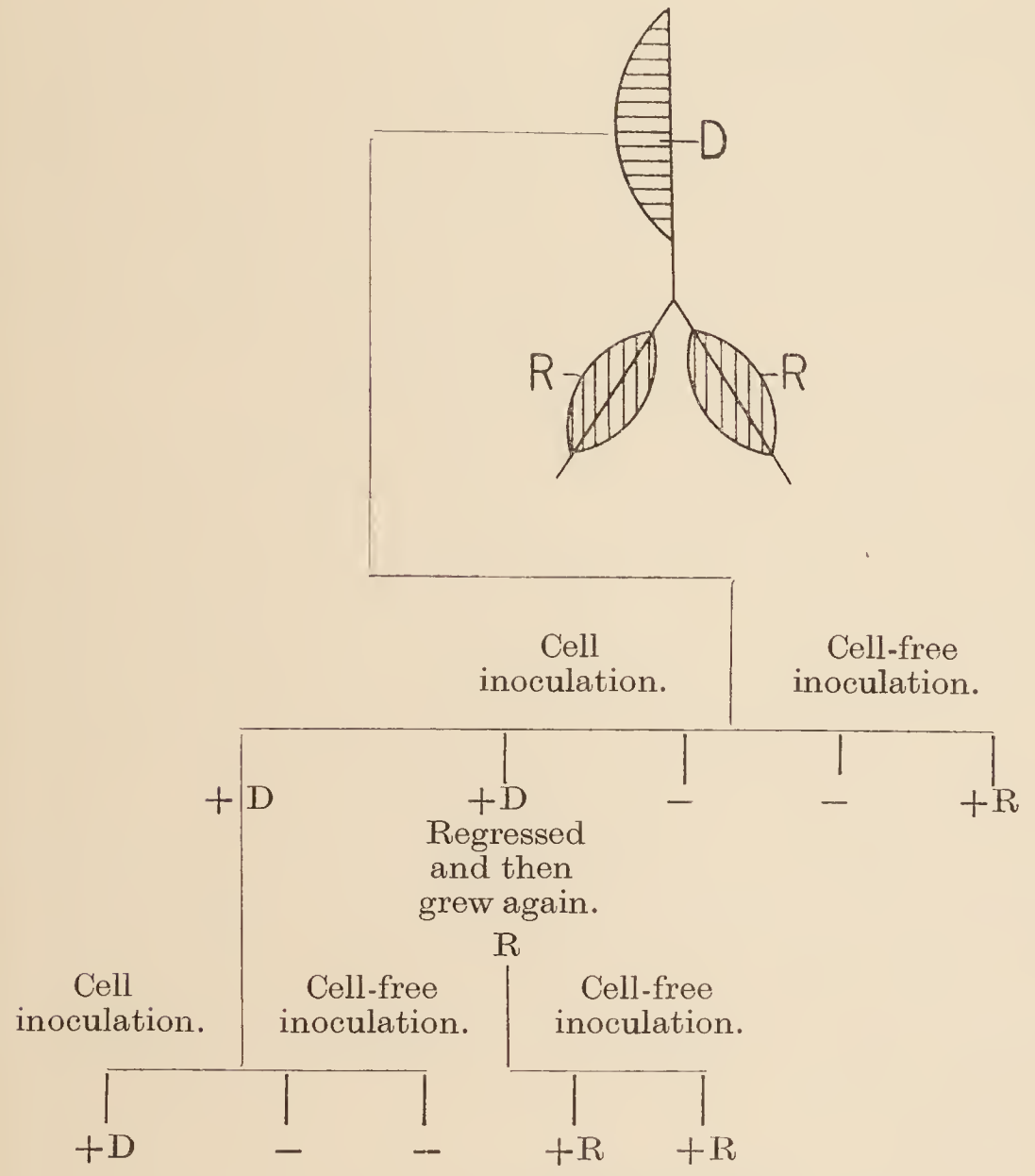
Of the many experiments carried out, expts. C and D are the only two in which it has been possible to show the presence of the Rous agent in the second generation of chemically induced tumours by direct experiment.

Regression of dibenzanthracene tumour and replacement by Rous sarcoma.

On two occasions an occurrence worthy of mention has happened. Dibenzanthracene tumours of the second generation, *i.e.* tumours grown from the cells of dibenzanthracene tumours of fowls also carrying Rous tumours, after showing good growth, regressed and after regression (in one case complete regression) other tumours grew in their place. The second tumours were typical Rous sarcomata in appearance and were readily propagated by cell-free filtrates. The following experiment illustrates this point.

Experiment E. A fowl was inoculated into the right breast muscle with dibenzanthracene tumour tissue (FD/2). After seven days it was inoculated into both legs with Rous cells and 14 days later it was killed, when it had fairly big tumours in the right breast and in both legs. Cells and cell-free filtrates of the dibenzanthracene tumour in the breast were then inoculated into other fowls and produced tumours. The tumour grown from the cell-free filtrate was typically Rous in appearance. Of the two birds inoculated with cells from the original dibenzanthracene tumour, one was of typically dibenzanthracene structure and could only be propagated by cells, not by cell-free filtrates. The second developed

tumours in both breasts, which regressed, that on the left side completely. The tumour in the right breast did not completely disappear but started to grow again. At death, this right breast tumour was of the Rous type and was propagated easily by cell-free filtrates. The following diagram illustrates these points.



EXPERIMENT E.

The changes in the breast dibenzanthracene tumour and its replacement by a Rous tumour can be seen in the following table.

		4 days after inoculation.	12 days.	20 days.	31 days.	34 days.	41 days.
			Dibenzanthracene tumour.				Rous tumour.
Inoculation with cells of dibenzanthracene tumour carrying Rous virus	Right breast	Neg.	++	++	+	?	++
	Left breast	Neg.	+	++	+	?	Neg.

In another experiment where the same kind of change occurred, both dibenzanthracene tumours in the breast muscles completely

disappeared, as far as palpation could indicate this, and other tumours grew which proved to be Rous sarcomata and could easily be propagated by cell-free filtrates.

It is important to note that in both of these experiments the original dibenzanthracene tumours in the breasts regressed, in one case completely, before new Rous tumours grew at the same site. This indicates that the growth of the Rous cells did not kill the dibenzanthracene cells, but it does not exclude the possibility that the Rous agent killed the dibenzanthracene tumour cells. This, however, seems unlikely, for dibenzanthracene tumours in fowls occasionally regress in any case, and there did not appear to be any increase in the incidence of regression of chemically induced breast tumours when associated with Rous tumours in the legs of the same bird as compared with those growing alone.

An attempt was made to obtain direct evidence on this point by injecting large quantities of Rous agent either into dibenzanthracene tumours or into the blood stream of fowls bearing these tumours. The Rous filterable agent did not make the dibenzanthracene tumours regress, but in some of the birds in which it was injected directly into the dibenzanthracene tumour, Rous tumour tissue was produced and the final tumours seemed to be a mixed growth of Rous and dibenzanthracene tissue.

On the whole, therefore, it appears that in the experiments described above in which dibenzanthracene tumours regressed and were replaced by Rous tumours, the regression was not due to the presence in them of Rous virus, but that the process of regression liberated the Rous virus from the cells and concentrated it sufficiently to allow the transformation of connective tissue cells to Rous sarcoma cells.

It seems possible that the transmission of Rous virus to the second generation of chemically induced tumours may be more common than the direct experimental evidence indicates; for, as mentioned above, only in two instances has it been possible by direct inoculation to obtain evidence of the presence of Rous agent in the second generation of dibenzanthracene and tar tumours. Had larger quantities of cell-free filtrate been used in these experiments, more positive results might have been obtained, but it has been the aim throughout this work to use the same technique and to inject approximately the same quantity of cell inoculum and of cell-free filtrate in all experiments (see p. 448).

Discussion.

It has been shown that where a fowl carries both chemically induced and Rous tumours at the same time, the filterable agent of the latter is transmitted to and taken up by the former, so that a cell-free filtrate of a dibenzanthracene or tar tumour may give

rise to a Rous sarcoma, while a cell inoculation of the dibenzanthracene or tar tumour will produce another chemical tumour of the same type. Generally it is only possible to obtain the two types of tumour from the chemically induced growth which has been associated with the Rous sarcoma in the same fowl. In two of the experiments described above, however (expts. C and D), cell-free filtrates from the second generation of two dibenzanthracene tumours and a tar tumour, which themselves had not had the opportunity of being directly infected by Rous filterable agent from a Rous tumour in the same bird, gave rise to Rous sarcomata, while the cells themselves gave rise respectively to dibenzanthracene tumours and a tar tumour of the third generation which could not be propagated by cell-free filtrates.

Other indirect evidence of the presence of the Rous agent in the second generation of dibenzanthracene tumours is afforded, however, by the fact that when regression of such a dibenzanthracene tumour takes place, a Rous sarcoma may grow at the same site. Thus it appears that although there may not be sufficient Rous agent in the second generation of a dibenzanthracene tumour to prove its presence by direct inoculation into another fowl, regression of the tumour may concentrate the Rous agent to a degree which allows it to become effective and to give rise to a Rous tumour, the latter then replacing the regressed dibenzanthracene tumour.

No evidence has been obtained that a dibenzanthracene tumour of the third or later generation carries the Rous virus.

It will be asked whether the filterable agent after transmission from the Rous tumour and deposition in the chemical tumour lies there inert or whether it multiplies. This question is difficult to answer, but the power of the Rous agent to multiply under these conditions seems limited except when favourable circumstances arise, such for instance as regression of the chemically induced tumour.

Even in the first generation of dibenzanthracene and tar tumours, *i.e.* those grown in the same fowl as Rous sarcoma, the actual amount of filterable agent transmitted by the Rous tumour and retained seems small as compared with the virus in a Rous tumour itself. This is evident from the fact that, when successful, only one or at most two fowls in three develop Rous tumours on injecting the ordinary amount of a cell-free filtrate of a dibenzanthracene tumour which has grown in fowls also carrying Rous tumours. In some experiments no Rous tumour is produced under the conditions described. Again, even when Rous tumours are produced by cell-free filtrates made from such dibenzanthracene tumours, the new tumours are slow in appearing, being usually only palpable 30 days or so after the injection. Once they begin to grow, the rate of development is, as a rule, as rapid as that of

ordinary Rous sarcomas. Normally, by the routine methods adopted in this laboratory, a cell-free filtrate of a Rous growth produces a palpable tumour in about 14 days. These facts suggest that the Rous filterable agent transmitted from a Rous sarcoma to a dibenzanthracene tumour is attenuated, and that its power to multiply in its new habitat is limited.

On the other hand it seems probable that the amount of Rous agent demonstrable in the second generation of dibenzanthracene and tar tumours can only be accounted for by its actual reproduction in this tumour. If this be the case, evidence of its presence in the third and later generations of dibenzanthracene tumours might be expected, but no such evidence has been obtained. When a Rous tumour begins to grow and replaces the regressed dibenzanthracene tumour, there is abundant production of the Rous agent. But the point at issue is whether the Rous agent multiplies in the dibenzanthracene tumour independently of Rous cells. It may be true that when the Rous agent is present in the second generation of chemically induced tumours, some Rous tissue is present and the tumour is a mixed one. Whether this is the case cannot be stated with certainty, but if this is the explanation, the amount of Rous tissue must be small. The question, therefore, whether the Rous agent increases in amount while present in the dibenzanthracene or tar tumour independently of Rous cell formation cannot be answered on the basis of present knowledge.

In a later paper, evidence will be given to show that most if not all of the normal organs of a fowl bearing a Rous sarcoma become invaded by the Rous agent in varying degrees, but are unaffected by it.* The same fact seems to hold as regards the dibenzanthracene and tar tumours. They also become a receptacle for the Rous filterable agent but are apparently not otherwise affected by it. There is, however, as will be seen later, one big difference between a normal, chemically induced tumour of a fowl and the spleen or liver of a fowl carrying the Rous agent. Injection of liver or spleen *cells* containing Rous virus into another fowl produces a Rous sarcoma, whereas injection of the dibenzanthracene or tar tumour cells, although containing Rous virus, will produce a dibenzanthracene or tar tumour. Cell-free filtrates of the same liver, or spleen, or of dibenzanthracene or tar tumours will all produce Rous sarcomata.

These experiments show that a chemically induced tumour can be infected by a cancer virus agent and to that extent support the view that McIntosh's results may well have been due to such an infection in his fowls carrying tar tumours. The results in

* Reference was made to these experiments in the 12th and 13th Annual Reports of the British Empire Cancer Campaign (1935 and 1936).

themselves, however, give no support to the suggestion that chemically induced tumours in fowls normally contain a filterable active agent. Support for this latter view has recently been obtained by Andrewes (1936). He found that a tar tumour of a fowl, originally produced in the University Field Laboratory, Sheffield, grew on occasion when cells were inoculated into adult pheasants. Precipitin tests appeared to show that the tumour in one pheasant consisted of pheasant cells. All the pheasants inoculated with the tar tumour developed antibodies which neutralised the virus of Rous sarcoma no. 1. Since such antibodies have never yet been found in normal pheasant sera and could not be induced by immunisation of pheasants with normal fowl embryo, it seemed probable that the neutralisation of the Rous agent was caused by anti-viral and not by anti-fowl bodies. Thus Andrewes drew the conclusion that, although neither at Sheffield nor in his own hands could direct evidence be obtained that the Sheffield tar tumour contained a filterable agent—*i.e.* no tumour could be produced in fowls by a cell-free filtrate of the tar tumour, yet the indirect evidence indicated its presence.

My own experience, formed on the basis of the work described in this paper and of other experiments, is that chemically induced tumours and virus tumours are essentially different in their nature and largely independent of and unaffected by each other. The initiating agent of growth in the dibenzanthracene and tar tumours seems to lie in their intact cells and these do not appear to be changed or even influenced by the Rous agent. The initiating agent of the Rous tumour is its filterable active factor, and this again seems to be uninfluenced by the cells of the chemically induced type of tumour. At the same time I must admit that these deductions are contrary to everything I expected and the discovery of a link connecting the initiating factors in the two types of tumour would no doubt alter the interpretation of the experimental results described.

Summary.

1. When a fowl carries at the same time tumours of the type induced by chemical agents such as dibenzanthracene or tar and other tumours of the Rous or filterable agent type, the Rous factor passes into the chemically induced tumour but leaves it apparently unaffected.

2. Injection of the cells of such a chemically induced tumour into a fowl produces a tumour of a structural type which can only be further propagated by cell inoculation.

3. Injection into other fowls of a cell-free filtrate of such a chemically induced tumour in a fowl bearing also a Rous sarcoma, if it produces a tumour at all, produces one of the Rous type.

4. In two out of many experiments, *cell-free filtrates* made from the second generation of chemically induced tumours—*i.e.* those which themselves had no association with a Rous sarcoma in the same fowl—produced a Rous sarcoma (3rd generation) which could be further propagated by cell-free filtrate. *Cells* of these chemically induced tumours, however, produced other (3rd generation) tumours of the same type as those originally chemically induced and these gave no evidence of containing the Rous agent.

5. In two experiments, second generation dibenzanthracene tumours of fowls regressed and new tumours grew in their place. These new tumours were Rous in character and could be readily propagated by cell-free filtrates. There is no good reason to believe that the presence of the Rous agent actually made the dibenzanthracene tumours regress, as such regression may happen without the presence of Rous sarcoma in the same bird. Injection of Rous filterable agent into a dibenzanthracene tumour will not make it regress, but may produce Rous sarcomatous tissue in it. It appears however, that when regression took place, the Rous agent exerted its effect and produced a second Rous tumour replacing the dibenzanthracene tumour.

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